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Development of a polymerase chain reaction assay for the specific identification of *Burkholderia mallei* and differentiation from *Burkholderia pseudomallei* and other closely related Burkholderiaceae☆

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Abstract

Burkholderia mallei and *Burkholderia pseudomallei*, the etiologic agents responsible for glanders and melioidosis, respectively, are genetically and phenotypically similar and are category B biothreat agents. We used an in silico approach to compare the *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 genomes to identify nucleotide sequences unique to *B. mallei*. Five distinct *B. mallei* DNA sequences and/or genes were identified and evaluated for polymerase chain reaction (PCR) assay development. Genomic DNAs from a collection of 31 *B. mallei* and 34 *B. pseudomallei* isolates, obtained from various geographic, clinical, and environmental sources over a 70-year period, were tested with PCR primers targeted for each of the *B. mallei* ATCC 23344-specific nucleotide sequences. Of the 5 chromosomal targets analyzed, only PCR primers designed to $bimA_{Bm}$ were specific for *B. mallei*. These primers were used to develop a rapid PCR assay for the definitive identification of *B. mallei* and differentiation from all other bacteria.

Keywords: *Burkholderia*; Glanders; Melioidosis; PCR

1. Introduction

Both *Burkholderia mallei* and *Burkholderia pseudomallei* are closely related Gram-negative bacterial pathogens that have the capability to cause severe human and animal disease. *B. mallei*, the causative agent of glanders disease, is a host-adapted equine pathogen that has been shown to be a clone of *B. pseudomallei* (Godoy et al., 2003; Waag and DeShazer, 2004). *B. mallei* primarily infects horses, donkeys, and mules, whereas humans are considered an incidental host. With the development of motorized transportation in the early 20th century and implementation of quarantine precautions for imported animals, no naturally occurring human cases of glanders have been reported in the United States since the 1930s (Waag and DeShazer, 2004). However, there are sporadic incidences that still occur in Asia, the Middle East, South America, and Africa. Human glanders occurs in individuals such as veterinarians, slaughterhouse workers, and laboratory scientists whose occupation exposes them to the pathogen. In solipeds, 2 distinctive forms of glanders may arise: acute (observed in mules and donkeys) and chronic (common in horses). Like solipeds, both acute and chronic forms of glanders can exist in humans, depending on the route of exposure (i.e., aerosol versus cutaneous). Human acute

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14. ABSTRACT

Burkholderia mallei and Burkholderia pseudomallei, the etiologic agents responsible for glanders and melioidosis, respectively, are genetically and phenotypically similar and are category B biothreat agents. We used an in silico approach to compare the B. mallei ATCC 23344 and B. pseudomallei K96243 genomes to identify nucleotide sequences unique to B. mallei. Five distinct B. mallei DNA sequences and/or genes were identified and evaluated for polymerase chain reaction (PCR) assay development. Genomic DNAs from a collection of 31 B. mallei and 34 B. pseudomallei isolates, obtained from various geographic, clinical, and environmental sources over a 70-year period, were tested with PCR primers targeted for each of the B. mallei ATCC 23344-specific nucleotide sequences. Of the 5 chromosomal targets analyzed, only PCR primers designed to bimA(Bm) were specific for B. mallei. These primers were used to develop a rapid PCR assay for the definitive identification of B. mallei and differentiation from all other bacteria.

15. SUBJECT TERMS
Burkholderia mallei, pseudomallei, genomic analysis, comparison, PCR, actin polymerization

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glanders is characterized by fever and fatigue as well as inflammation of and nodule formation on the face and peripheral limbs (Neubauer et al., 1997). Chronic glanders in humans presents with swollen lymph nodes, ulcerating nodules in the alimentary and respiratory tracts, weight loss, and numerous subcutaneous abscesses. Human glanders is a lethal disease that is essentially indistinguishable from human melioidosis (caused by *B. pseudomallei*), and if appropriate antibiotics are not administered, death normally occurs (Neubauer et al., 1997; Waag and DeShazer, 2004). Melioidosis, caused by the Gram-negative bacterium *B. pseudomallei*, is endemic in tropical regions throughout the world with the most prevalent regions being Southeast Asia and northern Australia (Cheng and Currie, 2005). Sporadic cases of melioidosis have been reported in the Indian subcontinent, Central and South America, the Caribbean, Africa, Iran, the Pacific Islands, and France in the early 1970s (Dance, 2002). *B. pseudomallei* is a facultative intracellular bacterium that is considered to be an opportunistic pathogen for humans, in particular, individuals with underlying risk factors including diabetes mellitus, alcoholism, and renal complications (Woods et al., 1999). *B. pseudomallei* can be acquired from contaminated environmental samples through inhalational or cutaneous routes of infection (Cheng and Currie, 2005). Symptoms of melioidosis are much like those of glanders and may include acute or chronic pneumonia, acute septicemia, and latent infections that can persist for years.

Because of the potential for *B. mallei* and *B. pseudomallei* weaponization, the rapid and definitive identification of these highly infectious pathogens is essential for the immediate initiation of appropriate antibiotic therapy. The current methods for diagnosing *B. mallei* and *B. pseudomallei* incorporate various biochemical and substrate utilization tests, cellular and colony morphology analysis, and motility assays, which can take up to 1 week to complete (Cheng and Currie, 2005; Neubauer et al., 1997). Furthermore, relying on biochemical testing alone for the identification of *B. mallei* and *B. pseudomallei* using readily available commercial kits (API 20NE and RapID NF) has recently been shown to be 0–60% accurate, at best (Glass and Popovic, 2005). These commercial systems may also falsely identify other organisms as *B. pseudomallei* and *B. mallei*.

Considering the high level of genetic, biochemical, and phenotypic similarities between *B. mallei*, *B. pseudomallei*, and *Burkholderia thailandensis* (Brett et al., 1997; Godoy et al., 2003; Holden et al., 2004; Nierman et al., 2004), molecular and biochemical approaches for identifying and differentiating these closely related *Burkholderia* species are problematic. There have been several reports in the literature that have targeted the *B. pseudomallei* 16S and 23S rRNA, 16S-23S intergenic region, flagellin C (*fliC*), heat shock protein 70, and a type 3 secretion (TTS) system for the molecular identification of *B. pseudomallei* and possible differentiation from *B. mallei* (Antonov et al., 2004; Gee et al., 2003; Hagen et al., 2002; Lee et al., 2005; Sprague et al., 2002; Tanpiboonsak et al., 2004; Thibault et al., 2004; Tomaso et al., 2005; Tomaso et al., 2004; Tyler et al., 1995). Despite the numerous misleading titles in the literature (i.e., titles that imply *B. mallei*-specific), there are no *B. mallei*-specific molecular assays for discriminating this obligate mammalian pathogen from *B. pseudomallei*.

The recent availability of complete genome sequences for *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 (Holden et al., 2004; Nierman et al., 2004) has greatly facilitated the ability to identify unique *B. mallei* DNA sequences. Using comparative in silico DNA sequence analysis, we identified a unique nucleotide sequence that is highly conserved among all virulent *B. mallei* isolates. From these data, a *B. mallei*-specific polymerase chain reaction (PCR) assay was developed and tested for specificity against a panel of diverse clinical and environmental isolates of *B. pseudomallei* and other closely related Gram-negative species.

2. Materials and methods

2.1. Bacterial strains used in this study

An extensive representative panel of 31 *B. mallei*, 34 *B. pseudomallei*, and 12 *B. thailandensis* strains isolated from various geographic, clinical, and environmental locations throughout the world were investigated (Table 1). Additional *Burkholderia* species tested in this study include *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia multivorans*, and *Burkholderia vietnamiensis*. Other Gram-negative and Gram-positive isolates analyzed for cross-reactivity with the *B. mallei*-specific assay include *Escherichia coli*, *Chromobacterium violaceum*, *Yersinia pestis*, *Pseudomonas aeruginosa*, and *Bacillus anthracis*. Genomic DNA for PCR amplification was purified using previously described methods (Wilson, 1987), and template DNA for *Bacillus anthracis* was kindly provided by Dr Donald Chabot, United States Army Medical Research Institute of Infectious Diseases. All *B. mallei* isolates were cultured using Luria–Bertani (LB) broth containing 4% glycerol (LBG) (Sigma, St. Louis, MO). With the exception of *Y. pestis*, which was grown on sheep blood agar plates (agar plate scrapings were used for genomic DNA purification), all other strains used in this work were grown in LB broth. *C. violaceum* and *Y. pestis* were propagated at 25 °C, whereas the remaining bacterial isolates were cultured at 37 °C with aeration.

2.2. In silico genomic subtraction

Using a combination of Critica and Glimmer, total open reading frames (ORFs) of *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 were overpredicted from 2003 draft versions of their genomic sequences. The approximately 6200 *B. mallei* ORFs were compared to those of *B. pseudomallei* using BLASTP (Ver. 2.1; web interface: low complexity filter, off; E-value cutoff, 100; -b, 1; -v, 1).

Table 1
Bacterial strains and their reactivity with 5 PCR primer pairs used in the study

Species and strain	Source	Location	Year	BMAA0749 (*bimA*$_{Bm}$)[a]	ISBma*4*[a]	BMAA0610[a]	BMAA0611[a]	BMA0860[a]
B. mallei								
2002721274	UN	US	1956	+	+	–	+	+
2002721273	UN	US	1956	+	+	+	–	+
2002721776	UN	US	1956	+	+	+	–	+
2002721277	UN	US	1956	+	+	+	+	+
2002721279	Human	US	1964	–	+	+	+	+
2002721280	UN	France	1972	+	+	+	+	+
2002721278	Human	US	1964	–	+	+	+	+
2000031064	UN	India	UN	+	+	–	+	+
2000031065	UN	Turkey	UN	+	+	–	–	+
2000031066	UN	India	UN	+	+	–	+	+
Turkey 1	UN	Turkey	UN	+	+	+	–	+
Turkey 2	UN	Turkey	UN	+	+	–	+	+
Turkey 3	UN	Turkey	UN	+	+	+	+	+
Turkey 4	UN	Turkey	UN	+	+	+	+	+
Turkey 5	UN	Turkey	UN	+	+	+	+	+
Turkey 6	UN	Turkey	UN	+	+	–	+	+
Turkey 7	UN	Turkey	UN	+	+	–	+	+
Turkey 8	UN	Turkey	UN	+	+	–	–	+
Turkey 9	UN	Turkey	UN	+	+	+	+	+
Turkey 10	UN	Turkey	UN	+	+	+	+	+
NCTC 120	UN	UK	1920	+	+	+	+	+
NCTC 10248	Human	Turkey	1950	+	+	–	–	–
NCTC 10229	UN	Hungary	1961	+	+	+	+	+
NCTC 10260	Human	Turkey	1949	+	+	+	+	+
NCTC 10247	UN	Turkey	1960	+	+	+	+	–
NCTC 3708	Mule	India	1932	+	+	+	+	+
NCTC 3709	Horse	India	1932	+	+	–	+	+
ATCC 23344	Human	China	1942	+	+	+	+	+
ATCC 10399	Horse	China	1956	+	+	+	+	+
ATCC 15310	Horse	Hungary	1961	+	+	+	+	+
ISU	UN	UN	UN	+	+	+	+	+
B. pseudomallei								
238	Blood	Australia	UN	–	+	–	–	+
295	Soil	Australia	UN	–	+	+	+	+
713	Ulcer	Australia	UN	–	+	+	+	+
439a	Ulcer	Australia	UN	–	+	+	+	+
112c	Blood	Australia	UN	–	+	–	–	+
E24	Soil	Thailand	UN	–	+	+	+	+
465a	Blood	Australia	UN	–	–	+	+	+
1026b	Blood	Thailand	UN	–	–	+	+	+
E25	Soil	Thailand	UN	–	–	+	+	+
730	Ulcer	Australia	UN	–	–	+	+	+
503	Soil	Australia	UN	–	–	+	+	+
296	Soil	Australia	UN	–	–	–	+	+
776	Blood	Australia	UN	–	–	+	+	+
487	Ulcer	Australia	UN	–	–	+	+	+
644	Ulcer	Australia	UN	–	–	–	+	–
E8	Soil	Thailand	UN	–	+	+	+	+
E13	Soil	Thailand	1991	–	–	+	+	–
E12	Soil	Thailand	UN	–	+	+	–	–
506	Soil	Australia	UN	–	–	–	–	+
423	Blood	Australia	UN	–	–	+	–	+
K96243	Human	Thailand	1996	–	–	–	–	–
STW 199-2	Water	Thailand	UN	–	–	+	–	–
STW 35-1	Water	Thailand	UN	–	–	+	+	+
STW 176	Water	Thailand	UN	–	–	+	+	–
STW 152	Water	Thailand	UN	–	–	+	+	+
STW 115-2	Water	Thailand	UN	–	+	+	–	–
STW 102-3	Water	Thailand	UN	–	–	+	+	+
E203	Soil	Thailand	1992	–	+	+	+	+

(*continued on next page*)

Table 1 (*continued*)

Species and strain	Source	Location	Year	BMAA0749 (*bimA*$_{Bm}$)[a]	ISBma*4*[a]	BMAA0610[a]	BMAA0611[a]	BMA0860[a]
WRAIR 1188	UN	Thailand	1964	–	+	+	+	+
316c	Blood	Thailand	1987	–	–	–	–	–
Pasteur 52237	UN	Thailand	1964	–	–	+	+	+
USAMRU 32	UN	Malaysia	1964	–	+	+	+	+
576	Human	Thailand	UN	–	–	+	+	+
275	Soil	Australia	UN	–	–	–	+	+
B. thailandensis								
E111	Environment	Thailand	UN	–	+	+	+	–
E125	Environment	Thailand	1991	–	+	+	+	–
E27	Environment	Thailand	UN	–	+	+	+	–
E135	Environment	Thailand	UN	–	+	+	+	–
E96	Environment	Thailand	UN	–	–	+	–	–
E32	Environment	Thailand	UN	–	–	+	–	+
E30	Environment	Thailand	UN	–	–	+	–	–
E264	Environment	Thailand	1993	–	+	+	+	–
E100	Environment	Thailand	1991	–	–	+	–	+
E132	Environment	Thailand	UN	–	–	+	–	+
E105	Environment	Thailand	UN	–	+	–	+	+
E120	Environment	Thailand	UN	–	+	+	+	+

UN = unknown.

[a] Correspond to the genes tested for *B. mallei* specificity in the investigation.

Using the perl script flanders3.pl (M. Schell, unpublished data), 1 best-hit alignment pair for each *B. mallei* ORF was recovered from the pairwise, nongraphical view output file and a "homology score" for each calculated from alignment statistics. This homology score was calculated as: (amino acid identity between query and hit)^2 × (hit length/query length) × (alignment length/query length). All *B. mallei* ORFs with homology scores less than 0.22 were extracted by flanders3.pl and then compared to the genomic DNA sequence of *B. pseudomallei* (GenBank accession NC_006350/006351) using TBLASTN (low complexity filter, on; E-value cutoff, 10^{-11}, -v, 1; -b, 1). The TBLASTN output in hit-table format was processed with flanders3.pl and the 14 *B. mallei* ORFs with homology scores less than 0.22 were recovered. Each ORF was then analyzed by TBLASTN against the recently published *B. mallei* genome sequence (NC_006348/006349) and GenBank number to determine its genome coordinates and confirm its "uniqueness" to *B. mallei* ATCC 23344. Three of these ORFs were very small (<120 bp) and had DNA sequences that were not found in the final *B. mallei* genome assembly and hence were discarded. Nucleotide sequence alignments were performed with GeneJockeyII software (Biosoft, Cambridge, England) for Macintosh.

2.3. PCR design and limit-of-detection studies

With the use of nucleotide sequences from the ORFs recovered in our in silico genome analysis, the following PCR primers were designed: AT5 5′-TTCGATC-GATTCCTGCTATC-3′ and AT4 5′-GCGTTAAACGCCG TACTTTC-3′ (*bimA*$_{Bm}$), 1027F 5′- CGCGCGCAGGT ACTCAACTTC-3′ and 1027R 5′-GATGGATTACG GCGCAAAGGG-3′ (ISBma*4*), 610F 5′-CGCGTCG GGCCGGCAATCGTGTG-3′ and 610R 5′-CCGGT GCTCGCGTTCGCCATCTCG-3′ (BMAA0610), 611F 5′ GCCCGAGCCCGCGAATCACC- 3′ and 611R 5′ GGCGACACGACGACGAACGGAATC-3′ (BMAA0611), and mntHF 5′-CATCATGGATGGCTTT-CTGC-3′ and mntHR 5′-TCGTTATGCTAACCAG-GACG-3′ (BMA0860). PCR amplification targeting a hypothetical protein (BMAA0732) encoded by both *B. mallei* and *B. pseudomallei* was performed using primers 0732F 5′-TGAAGCTGACCGATTCGATGATGC-3' and 0732R 5′-TCAGATAGAGCGACAGCAGGATGG-3′ to confirm the integrity of our *B. pseudomallei* genomic DNA preparations. PCR amplification for each genomic target was performed using the following parameters: 1 cycle at 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, followed by a final 7-min extension at 72 °C with the Epicentre FailSafe kit using buffer "J" (Epicentre Technologies, Madison, WI). PCR reactions were composed of the following components: 2.5 μL of the forward and reverse primers (20 μM), 5 μL of template DNA (100 ng), 14 μL of sterile water, 25 μL of 2× reaction buffer J, and 1 μL of Taq DNA polymerase (2.5 U). Reactions were analyzed (10 μL) on a 2.0% agarose gel containing ethidium bromide (Sambrook et al., 1989).

A 3-mL LBG culture was inoculated with *B. mallei* (50 μL from a glycerol stock) and incubated for 18 h at 37 °C to determine if our *B. mallei*-specific PCR assay was capable of detecting crude DNA preparations. Serial 10-fold dilutions of the overnight culture (100 μL of the bacteria into 900 μL of phosphate-buffered saline [PBS]) were made to obtain cell concentrations ranging from 8.4×10^8 to 8.4×10^1 CFU/mL. A duplicate dilution series was made for bacterial counts. Each 10-fold dilution was centrifuged

for 5 min at 14 000 rpm using a Spectrafuge (E&K Scientific, Campbell, CA), and the cell pellets were resuspended in 100 μL of PBS and heated at 100 °C for 10 min. Cellular lysates were centrifuged for 5 min at 14 000 rpm using a benchtop Spectrafuge to remove cellular debris, and 10 μL of each supernatant was used for PCR analysis as previously described.

For limit-of-detection analysis, a 990 ng/μL stock of *B. mallei* ATCC 23344 genomic DNA was serially diluted to obtain working stocks ranging from 1 μg to 1 fg. For each PCR reaction, 5 μL of template DNA was used as described above.

3. Results

3.1. Gene targets used for assay development

Using our in silico approach, a total of 11 *B. mallei* ATCC 23344 ORFs were found to be completely missing from the *B. pseudomallei* K96243 genome. However, 7 of these were found to be present (>75% amino acid identity) in the genomes of other sequenced *Burkholderia* sp., leaving only 4 as candidates for *B. mallei*-specific gene sequences (ISBma*4*, BMAA0610, BMAA0611, and BMA0860).

ISBma*4* is an IS*3* family insertion sequence (IS) element that is found at 2 locations on chromosome 1 of *B. mallei* ATCC 23344, but is not present in the genome of *B. pseudomallei* K96243 (Holden et al., 2004; Nierman et al., 2004). The first copy is located at 998 950 through 999 495 and the second copy is at 1 467 508 through 1 468 017. Like many genes in the *B. mallei* chromosome, both copies of ISBma*4* are disrupted by the abundant IS*3* family element IS*407*A (Nierman et al., 2004). The PCR primers 1027F and 1027R were designed to amplify a 308-bp PCR product from ISBma*4* (Table 1). This sequence encodes an ORF with 52% amino acid identity to OrfA of insertion sequence IRS011 of *Ralstonia solanacearum* (gi17430114).

BMAA0610 encodes a di-haem cytochrome *c* peroxidase family protein and is not present on either of the *B. pseudomallei* K96243 chromosomes. BMAA0610 is encoded on chromosome 2 between bp 621 948 and 620 338. Likewise, an additional ORF, BMAA0611, located directly downstream from BMAA0610, is also unique to *B. mallei* ATCC 23344 and encodes a putative phosphoesterase family protein. BMAA0611 is located at positions 623 619–621 952 on chromosome 2. Comparative analysis of the region surrounding these genes in *B. pseudomallei* K96243 (chromosome 2) indicated that the genes are deleted from the *B. pseudomallei* genome rather than inserted into the *B. mallei* genome. There is a small nucleotide region encoded by *B. pseudomallei* that matches to the 3′ end of BMAA0611. The deletion of the 2 genes appears to have been completed by 2 closely located but separate deletion events, 1 of which deleted the BMAA0610 homolog completely and the second that removed most of the BMAA0611 homolog leaving a small remnant from the 3′ end of the gene. It is noteworthy that these genes were lost in *B. pseudomallei* K96243 relative to *B. mallei* ATCC 23344, which contains a smaller genome relative to *B. pseudomallei*. Genome reduction in *B. mallei* is believed to be mediated by IS elements (Nierman et al., 2004). However, for these predicted gene deletion events in the *B. pseudomallei* genome, no 4-bp insertion site duplication, characteristic of IS element-mediated deletions, was found at the deletion junctions, suggesting an alternative mechanism. PCR primers were designed to amplify small internal amplicons (381 and 402 bp) within BMAA0610 and BMAA0611 and tested for specificity against our *B. mallei* and *B. pseudomallei* culture collection, respectively (Table 1).

Another ORF detected in silico as specific to ATCC23344 is BMA0860 encoding a Mn^{2+}/Fe^{2+} transporter belonging to the Nramp family of metal transporters (Richer et al., 2003). It is on chromosome 1 at coordinates 899 711–901 024. Sequence alignments between BMA0860 and the corresponding *B. pseudomallei* K96243 chromosome region revealed extensive nucleotide divergence. This sequence heterogeneity between *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 was found within the coding region of BMA0860 in addition to the up- and downstream chromosomal segments (data not shown). In fact, a 995-bp region spanning from bp 887 through bp 1665 within BMA0860 was absent in the *B. pseudomallei* K96243 genome. With the use of this *B. mallei* ATCC 23344 unique sequence, PCR primers mntHF and mntHR were designed (amplicon of 284 bp) and tested for *B. mallei* specificity (Table 1).

The *Burkholderia* intracellular motility A gene ($bimA_{Bm}$, BMAA0749) was detected as a potential *B. mallei*-specific target during analyses of its role in actin-based motility and cell-to-cell spread (Stevens et al., 2005a). BimA is a putative type V autotransported protein (Henderson et al., 2004) with similarity at the carboxy-terminus to the *Yersinia enterocolitica* YadA and *Haemophilus influenzae* Hia autotransporters. *B. pseudomallei* also harbors a *bimA* allele ($bimA_{Bp}$) (Stevens et al., 2005a, 2005b). Although the terminal 300 bp of this gene is >99% identical to the 3′ end of *B. mallei* $bimA_{Bm}$ (Fig. 1), its 5′ region exhibited no nucleotide sequence similarities to the same region of $bimA_{Bm}$ (Fig. 1). In fact, the nucleotide sequence disparity extended to the 3′ termini of the upstream alleles (BPSS1491 and BMAA0750). The amino-terminal region of BimA is exposed at the bacterial cell surface and is thought to be involved in recruiting and polymerizing actin (Stevens et al., 2005a, 2005b). The genome of *B. thailandensis* E264, an avirulent *Burkholderia* species very closely related to *B. mallei* and *B. pseudomallei* (Brett et al., 1998), also encodes a $bimA_{Bt}$ allele with a unique 5′ region and a 3′ region (www.tigr.org) nearly identical to that of $bimA_{Bm}$ and $bimA_{Bp}$. PCR primer pairs AT4 and AT5, using the nucleotide sequence heterogeneity of the 5′ regions of $bimA_{Bp}$, $bimA_{Bm}$, and $bimA_{Bt}$, were designed to generate a *B. mallei*-specific amplicon of 250 bp (Fig. 2 and

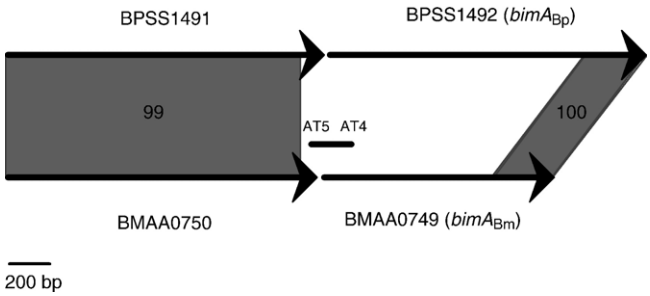

Fig. 1. Comparative analysis of the *bimA* loci of *B. pseudomallei* K96243 and *B. mallei* ATCC 23344. *B. pseudomallei* and *B. mallei* genes are depicted schematically at the top and bottom, respectively. The locations and directions of the transcription of the genes are represented by arrows. Shaded areas indicate DNA sequences that are highly conserved in both species, and the numbers in these areas indicate the percentage of nucleotide identity in these regions. The location of the *B. mallei*-specific PCR amplicon generated by primers AT4 and AT5 is shown schematically. The *bimA* alleles encode autotransported proteins involved in actin-based motility and cell-to-cell spread. BPSS1491 and BMAA0750 are putative heptosyltransferases that may modify the activity of BimA by posttranslationally attaching a heptose residue(s). A scale, in base pairs, is shown at the bottom.

Table 1). Although BMAA0749 was not selected as unique to *B. mallei* by our in silico genomic subtraction method, examination of the intermediate output revealed it had a homology score of 0.225, just slightly above the "uniqueness" cutoff score we applied.

3.2. Assay specificity

A total of 5 *B. mallei* ATCC 23344 unique nucleotide sequences/genes were used for the development of our PCR-based *B. mallei*-specific assay, which are designated by locus identifier ($bimA_{Bm}$, ISBma*4*, BMAA0610, BMAA0611, and BMA0860). A total of 31 *B. mallei* and 34 *B. pseudomallei* strains isolated from various clinical, environmental, and geographic locations, representing a collection period of approximately of 70 years, were used for specificity determination. In addition, a panel of 12 *B. thailandensis* strains, several *Burkholderia* sp., and several other Gram-negative bacteria were included to assess cross-reactivity.

The $bimA_{Bm}$ amplicon was present in 29 of 31 (94%) *B. mallei* strains, including all of the virulent isolates (Table 1 and Fig. 2). Two avirulent strains, *B. mallei* 2002721278 and *B. mallei* 2002721279, did not produce an amplicon with the AT4 and AT5 primer pair. Interestingly, none of 34 *B. pseudomallei* isolates tested produced a detectable $bimA_{Bm}$ amplicon, demonstrating the specificity of this *B. mallei* assay (Table 1). Likewise, the 12 *B. thailandensis* strains used in this study did not yield a PCR product with the AT4 and AT5 primer pair (Table 1). In addition, our Gram-negative reference panel, which included *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. multivorans*, *B. vietnamiensis*, *E. coli*, *C. violaceum*, *Y. pestis*, and *P. aeruginosa* also failed to yield PCR products with these primers (data not shown).

With the use of primers 1027F and 1027R, which targeted ISBma*4*, 31 of 31 (100%) *B. mallei* strains and 12 of 34 (35%) *B. pseudomallei* strains produced detectable amplicons (Table 1). In addition, 7 of 12 (58%) *B. thailandensis* strains yielded PCR products when using primers 1027F and 1027R (Table 1). No other species in our Gram-negative reference panel produced an amplicon with this primer pair (data not shown).

BMAA0610 was present in 21 of 31 (67%) *B. mallei* isolates, 26 of 34 (76%) *B. pseudomallei* isolates, and 11 of 12 (92%) *B. thailandensis* isolates (Table 1). Similarly, BMAA0611 was present in 25 of 31 (81%) *B. mallei* isolates, 25 of 34 (74%) *B. pseudomallei* isolates, and 7 of 12 (58%) *B. thailandensis* isolates (Table 1).

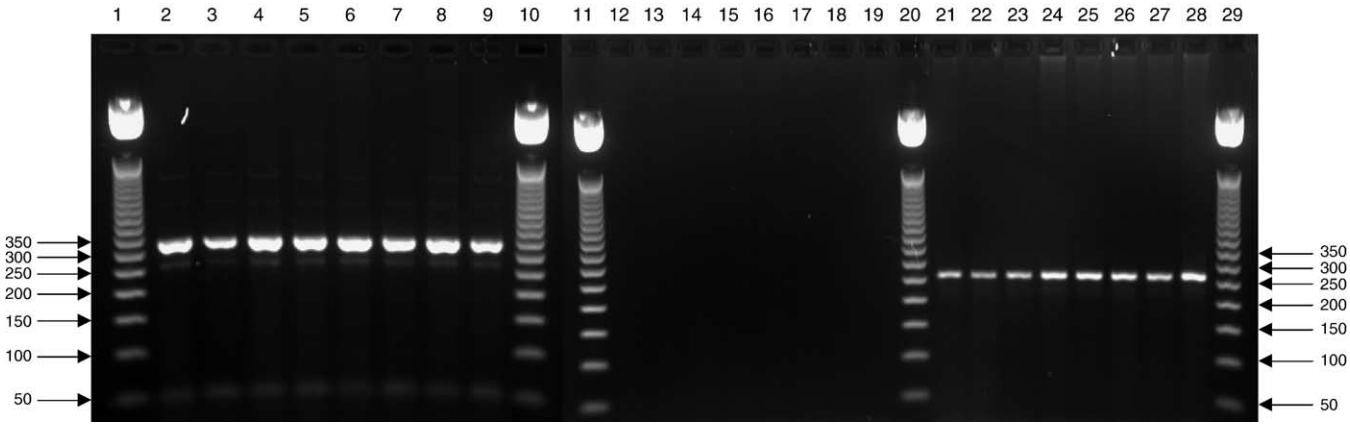

Fig. 2. Representative gel of PCR products using template DNA isolated from 8 *B. pseudomallei* and 8 *B. mallei* strains. Lanes 1, 10, 11, 20, and 29 contain a 50-bp stepladder (Promega, Madison, WI). Lanes 2–9 are control PCR reactions on *B. pseudomallei* genomic DNA preparations, which depict the integrity of the target DNA. The following *B. pseudomallei* strains were used and are positioned starting in lane 2: K96243, 487, USAMRU 32, 238, E25, STW 176, 316c, and 439a. Lanes 12–19 represent PCR reactions performed on *B. pseudomallei* strains (described above) using the *B. mallei*-specific PCR primers AT4 and AT5. Lanes 21–28 are *B. mallei*-specific amplicons obtained using primers AT4 and AT5. The following *B. mallei* isolates were used and are arranged beginning in lane 21: ATCC 23344, NCTC 10229, 2000031065, Turkey 8, Turkey 1, 2002721273, Turkey 10, and 2002721776. PCR products were separated on a 2% agarose gel containing ethidium bromide.

The *B. mallei* Mn^{2+}/Fe^{2+} Nramp transporter (BMA0860) appears to be relatively well conserved with only 2 *B. mallei* isolates failing to produce a detectable amplicon (Table 1). Moreover, 76% of the *B. pseudomallei* strains (but not K96243) and 42% of *B. thailandensis* strains also yielded an amplicon of the correct size with the mntHF and mntHR primer pair (Table 1).

Taken together, these results suggest that there is significant strain variability among these *Burkholderia* species, and it is difficult to identify species-specific sequences from a limited number of sequenced strains. This was clearly demonstrated by the fact that ISBma*4*, BMAA0610, BMAA0611, and BMA0860 were not present in the genome of *B. pseudomallei* K96243 (Holden et al., 2004), but were variably present in other *B. pseudomallei* strains. Given the fact that the DNA shared by *B. mallei* and *B. pseudomallei* is ~99% identical at the nucleotide sequence level (Holden et al., 2004; Nierman et al., 2004), it was surprising that the 5′ region of $bimA_{Bm}$ was found exclusively in *B. mallei*. The sequence was present in 94% of *B. mallei* strains tested and was absolutely conserved among virulent isolates of *B. mallei*. Based on these results, we developed a *B. mallei*-specific PCR assay using the AT4 and AT5 primer pair (Fig. 1).

3.3. Assay sensitivity

The sensitivity of our *B. mallei*-specific PCR assay was determined by serially diluting quantified *B. mallei* ATCC 23344 genomic DNA to obtain working stocks ranging from 1 μg to 1 fg. The limit of detection for the assay was approximately 10 ng, which is consistent with other *Burkholderia* PCR assays (Bauernfeind et al., 1998; Lee et al., 2005). PCR was performed on extracts of bacterial cells serially diluted in PBS as described in Materials and methods to determine if our assay would work on crude DNA preparations. As anticipated, primers AT4 and AT5 produced a single amplicon using crude bacterial lysates with a limit of detection of ca. 10^5 CFU/mL.

4. Discussion

Rapid and accurate diagnosis of *B. mallei* is essential for immediate prophylactic treatment, especially in cases of acute and/or septic glanders. The standard method for the diagnosis of *B. mallei* primarily relies on biochemical testing, which can take in excess of 7 days (Waag and DeShazer, 2004). Consequently, these approaches are less than satisfactory for patients with acute glanders as death normally occurs within 2 days. Furthermore, it has been shown that readily available commercial kits (API 20NE and RapID NF) used for *B. mallei* identification cross-react with other nonvirulent bacterial species leading to false-negative results (Glass and Popovic, 2005; Inglis et al., 1998). Likewise, and because of the antigenic relatedness between *B. mallei* and *B. pseudomallei*, there are currently no *B. mallei*-specific serologic assays (Waag and DeShazer, 2004). However, Burtnick et al. (2002) recently demonstrated that the lipopolysaccharide O-antigens of *B. mallei* and *B. pseudomallei* display differences in O-acetylation at position 4′ of L-talose. This finding probably explains the presence of species-specific LPS O-antigen epitopes and the existence of *B. mallei*-specific monoclonal antibodies (Anuntagool and Sirisinha, 2002) and bacteriophages (DeShazer, 2004; Woods et al., 2002). However, these observations have not been exploited for development of rapid, simple diagnostic assays.

Because of the rapid turnaround time, low concentration of starting target DNA needed, and specificity, PCR assays are becoming a popular means for bacterial identification and differentiation in many clinical and public health laboratories (Pitt et al., 2000). There are several reports in the literature that describe PCR-based assays for identifying *B. pseudomallei*; however, none of the assays are currently being used for melioidosis diagnosis (Bauernfeind et al., 1998; Gee et al., 2003; Hagen et al., 2002; Holden et al., 2004; Lee et al., 2005; Sprague et al., 2002; Tanpiboonsak et al., 2004; Thibault et al., 2004; Tomaso et al., 2004).

Recently, Tomaso et al. (2005) developed real-time PCR assays targeting the *B. pseudomallei* 16S rRNA, *fliC*, and the ribosomal subunit protein S21 (*rpsU*), none of which could distinguish *B. mallei* from *B. pseudomallei*. Likewise, Sprague et al. (2002) reported heterogeneity in the *fliC* gene from several *B. pseudomallei* isolates, which prevented the differentiation of *B. mallei* from *B. pseudomallei*. In addition, real-time PCR assays have been designed that target a conserved type 3 secretion system (TTS1, *orf*11 and *orf*13) encoded by both *B. mallei* and *B. pseudomallei* (Thibault et al., 2004). In the latter investigation, both *B. mallei* and *B. pseudomallei* were distinguished from *B. thailandensis* by amplification of *orf*13, but the assay was not successful at differentiating *B. mallei* from *B. pseudomallei*. Further, Antonov et al. (2004) attempted to target portions of the *B. mallei* and *B. pseudomallei* 23S rRNA to distinguish these species using standard PCR (not a real-time PCR platform) and reported that this highly conserved genomic target is not adequate for distinguishing *B. mallei* from *B. pseudomallei*.

In this study we used an in silico approach to screen the *B. mallei* ATCC 23344 and *B. pseudomallei* K96243 genomes to identify alleles unique to *B. mallei*. A total of 5 genes were targeted to develop a *B. mallei*-specific PCR assay, and each primer set was tested against an extensive panel of *B. mallei* and *B. pseudomallei* isolates recovered from various geographic, clinical, and environmental locations throughout the world. Of the 5 ORFs tested, only ISBma*4* was conserved among all 31 *B. mallei* isolates. However, numerous *B. pseudomallei* strains in our collection also contained this IS element, which suggests assays targeting ISBma*4* would produce false-positive reactions and would not be adequate for *B. mallei* detection (Table 1). Interestingly, the nucleotide sequence found in the 5′ end of BMAA0749, which encodes the $bimA_{Bm}$, was conserved

among all the virulent *B. mallei* isolates tested, but was not present in any *B. pseudomallei* isolates. Comparative genomic hybridization studies suggest that the 2 avirulent *B. mallei* isolates that did not amplify with the AT4 and AT5 primer pair both contain deletions that encompass BMAA0749 and flanking genes (H.S. Kim, unpublished data). It is unknown if the loss of $bimA_{Bm}$ is responsible for the loss of virulence in these strains or if this is due to the loss of the adjacent *virAG* (BMAA0745 and BMAA0746), a 2-component regulatory system known to be required for full virulence (Nierman et al., 2004). In any case, IS-mediated deletion events are common during laboratory cultivation of *B. mallei* (Nierman et al., 2004), and it is unlikely that these strains harbored these deletions when they were first isolated from their hosts. Our results demonstrate that all natural (virulent) *B. mallei* isolates contain $bimA_{Bm}$ and that the AT4 and AT5 primer pair will be extremely useful for the identification of *B. mallei*, and differentiation from *B. pseudomallei*, in the event this agent is used in a biologic attack.

Stevens et al., 2005b recently demonstrated that the *B. pseudomallei* BimA protein is required for actin-based motility in J774.2 cells. Interestingly, when *B. pseudomallei* is intracellular (i.e., within macrophages), this protein localizes at the pole of the bacteria where actin tails can be visualized. Disruption of the *B. pseudomallei* BimA inhibits host cell actin polymerization; however, heterologous expression of the *B. mallei* and *B. thailandensis bimA* in a *B. pseudomallei* $bimA^{-}$ strain fully restores this defective phenotype (Stevens et al., 2005a). The latter results suggest that despite the variability in nucleotide sequence between the $bimA_{Bm}$ and $bimA_{Bp}$, *B. mallei* clearly synthesizes a functional BimA protein. Using multilocus sequence typing (MLST), Godoy et al. (2003) recently proposed that *B. mallei* is a clone of *B. pseudomallei*. Our results suggest that this heterogeneity in the BimA protein occurred early in the divergence of *B. mallei* from *B. pseudomallei*. This hypothesis is further supported by the observation that each of the *B. mallei* strains tested in this study, with the exception of *B. mallei* 2002721278 and *B. mallei* 2002721279, produced a detectable amplicon with PCR primers targeting $bimA_{Bm}$, whereas each of our *B. pseudomallei* isolates did not (Table 1 and Fig. 2). The G + C content of the 3′ end of *bimA* in both species is 68% and the 5′ region is 70% in $bimA_{Bp}$ and 62% in $bimA_{Bm}$. The N-terminus of *B. mallei* BimA may have been horizontally acquired early in the evolution of *B. mallei* from *B. pseudomallei* and may have been retained because it was optimally suited for *B. mallei*'s lifestyle as an obligate mammalian pathogen.

Recent methods reported for the subtyping of *B. mallei* and *B. pseudomallei* include pulse-field gel electrophoresis, 16S rRNA sequencing, variable number tandem repeat polymorphisms, PCR–restriction fragment length polymorphism, and MLST, all of which are adequate for this type of analysis but are labor intensive and require several hours to complete (Godoy et al., 2003; Rantakokko-Jalava et al., 2000). In contrast, standard PCR reduces labor and requires significantly less time to obtain results. However, when incorporating this type assay for pathogen detection and differentiation, it is essential that the stability of the target sequence be evaluated. With the ability to specifically detect 29 of 31 *B. mallei* isolates (including all virulent isolates), and to differentiate these from *B. pseudomallei*, our findings demonstrate the unique nucleotide sequence identified at the 5′ end of $bimA_{Bm}$ is stable and provides an ideal target for the specific identification of virulent *B. mallei* isolates (Table 1 and Fig. 2). The results presented here also suggest that it should also be possible to design primers to the 5′ regions of $bimA_{Bp}$ and $bimA_{Bt}$ for the specific identification of *B. pseudomallei* and *B. thailandensis*, respectively.

By performing serial dilutions on purified genomic DNA we determined the limit of detection for our *B. mallei*-specific assay (using primer pairs AT4 and AT5 that target $bimA_{Bm}$) to be approximately 10 ng. Although this assay is not as sensitive as real-time PCR it still provides a rapid technique to specifically identify *B. mallei* and differentiate this pathogen from *B. pseudomallei*. In addition, this assay also successfully amplified the target DNA within $bimA_{Bm}$ using whole cell lysates of *B. mallei*, which suggest prior genomic DNA purification before PCR amplification is not necessary. It should be noted that primers AT4 and AT5 did not produce a $bimA_{Bm}$ amplicon when using DNA extracted from the lungs, spleen, and liver of a chronically infected female BALB/c mouse or from spiked blood. To determine if these results were a consequence of the limit of detection for the assay, we tested infected tissue extracts with a real-time PCR assay that targets both *B. mallei* and *B. pseudomallei*. As anticipated, *B. mallei* was detected in each tissue sample (data not shown). Further work will be needed to transition this *B. mallei*-specific PCR assay to a real-time PCR platform which will undoubtedly provide greater sensitivity and decrease turnaround time.

This investigation provides the first *B. mallei*-specific PCR assay capable of differentiating this highly infectious pathogen from *B. pseudomallei* and other closely related bacterial species. Our preliminary results incorporating 31 *B. mallei* isolates suggest that primer pair AT4 and AT5, which target a unique *B. mallei* nucleotide sequence, are ideal for rapidly and accurately identifying *B. mallei*.

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